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(54) Title: HAEMOSTATIC SPONGE (57) Abstract A haemostatic sponge comprising a porous structure of biologically absorbable, solid material, a haemostatically effective amount of thrombin or a precursor therefor, and one or more thrombin-stabilizing agents, said sponge having a total water content of below 50 % by weight, and a method for preparing a haemostatic sponge.		

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HAEMOSTATIC SPONGE

FIELD OF INVENTION

The present invention relates to a haemostatic sponge comprising a porous structure of biologically absorbable, solid material containing a haemostatically effective amount of thrombin, and one or more thrombin-stabilizing agents, said sponge having a water content of below 50% by weight; a method for preparing a haemostatic sponge as defined above; the use of such a dry thrombin-containing haemostatic sponge; and a method for arresting bleeding comprising the application to the site of bleeding of a haemostatic sponge.

TECHNICAL BACKGROUND

When blood vessels are injured by physical traumas including surgical interventions bleedings will occur. Dependent on the extent of the injury, bleedings may result in losses of blood which can affect the normal function of the individual or, in cases of bleedings occurring in osseous non-expandable cavities, the accumulation of extravasated blood may cause damages of soft tissues due to increased pressure. If bleedings are left alone they will eventually be arrested by a normally occurring physiological process characterized by a chain of events involving the combined activity of vascular, platelet, and plasma factors. This process is referred to as a physiological haemostasis an important element of which is blood coagulation which is described below. In the case of a minor superficial bleeding this physiological haemostasis is adequate for the arrest.

Blood coagulation may be described as occurring in steps:

(1) The formation of an activator of prothrombin, which is a precursor of the plasma serine protease thrombin. The prothrombin activator is a complex of an enzyme factor Xa, and 2 cofactors: factor Va and procoagulant phospholipids, both present on the surface of activated platelets. Furthermore, the presence of calcium ions is necessary for the function of the activator.

(2) The cleavage by the above activator system of prothrombin into two fragments, one of which is the enzyme thrombin.

(3) The conversion by thrombin of the plasma precursor fibrinogen to the clotting substance fibrin. This process involves several steps, the first of which comprises the cleavage of small peptides from fibrinogen, whereby fibrin monomers are formed, which then polymerize to form insoluble fibrin polymers. As a final step, thrombin activates the plasma factor XIII, an enzyme that catalyzes the formation of covalent bonds between fibrin molecules, thereby cross-linking the molecules to form a firm clot resistant to dissolution. When activated, factor XIII is designated factor XIIIa.

In the above step (1) leading to the formation of the prothrombin activator system, several plasma proteases are involved in a cascade of proteolytic events. These blood coagulation factors are currently referred to by using Roman numerals, such as factor VII, factor VIII, factor IX, factor XI, and factor XII. The cascade involves sequential proteolytic activations of the next enzyme in the cascade. Thus activated blood coagulation factors are designated by their Roman numerals followed by an "a", such as factor VIIa, factor VIIIa, or factor IXa.

However, bleedings emerging from more extensive injuries, especially such injuries which involve larger arteries or when seeping bleedings occur from larger mucosal surfaces or in cavities without drainage, require the adoption of surgical and/or medicamental haemostatic measures. Surgical arrest of bleeding comprise ligation or suture of disrupted blood vessels, plugging by using tampons in cavities, coagulating tissue surfaces including their exposed disrupted blood vessels by heated instruments or by the application of cauterizing agents or heated air.

Surgical haemostasis may also be aided by the application at the injured site of appropriately sized blocks, plates, or films of biologically absorbable haemostatic sponges. In this context, the term "sponge" is understood to mean a porous structure characterized in that the structure is reticulate and has an inner surface considerably larger than its outer surface, that it contains hollow

spaces within the reticulate structure, and that it can absorb many times its own weight in liquids.

"Biologically absorbable" is a term which in the present context is used to describe that the materials of which the said sponges are made can be degraded in the body to smaller molecules having a size which allows them to be transported into the blood stream. By said degradation and absorption the said sponge materials will gradually be removed from the site of application.

Such haemostatic sponges are useful for enhancing the arrest of bleedings in several instances of surgical interventions or other injuries such as in surgery of large abdominal organs (liver, spleen, or intestines); in lung surgery; in neurosurgery to prevent pressure damages of the cerebral or nerve tissues; in orthopedic surgery during which extensive haemorrhages frequently occur which are difficult to arrest by other means; in vascular surgery to arrest seeping bleedings from the sites of suturing; in oral or dental surgery such as extraction of teeth; and in nose-bleeding (epistaxis).

Materials for the preparation of haemostatic sponges are conventionally selected from biodegradable or biologically absorbable compounds such as collagen, gelatine, chitin, cellulose, polyglycolic acid and polylactic acid. Such absorbable haemostats can be left at the site of bleeding even after suturing of internal injuries and will exert their effect over a period of time, dependent on their water solubility, degradability, and size.

The characteristics of the above materials may be conditioned by various chemical or physical treatments resulting in e. g. a preferred improved mechanical strength of the structure or in rendering the material less water soluble thereby retarding the rate of absorption which may extend the period of haemostatic activity. As an example, gelatine may be denatured by treatment with an aldehyde such as formaldehyde or by thermal treatment at temperatures in the range of 100 - 160°C for several hours. After such treatment the originally water soluble gelatine will become substantially water insoluble but can still be degraded to absorbable molecules by proteo-

lytic enzymes present in the body. In contrast, haemostatic sponges prepared from undenatured gelatine will dissolve rather rapidly and turn into a soft gel when brought into contact with aqueous solutions or bleeding wounds.

- 5 The application of haemostatic sponges to injured sites may arrest bleeding by several mechanisms of action: a mechanically strong structure such as denatured gelatine may exert a plugging effect by compressing open blood vessels; blood platelets may become aggregated on the haemostat material and release their blood coagulation factors
10 which together with plasma factors lead to the formation of fibrin, the final coagulation product; platelets may also become activated to release their coagulation factors by the turbulent blood flows occurring within the meshes of the porous spongy structure.

- Pharmaceutical preparations containing thrombin or other blood coagulation factors such as factor VIII, factor XIII or calcium ions are
15 currently used as haemostatic adjuncts in surgery, said adjuncts being administered e. g. by spraying a suitable solution thereof onto the site of bleeding or by applying textile materials such as gauze or cotton wool fabrics thereon which prior to the application have
20 been soaked in a solution of one or more of said haemostatic compounds. Such procedures, however, implies considerable inconveniences: aqueous solutions has to be prepared from freeze-dried or frozen preparations just before use due to low stability when dissolved; microbial contamination of the solution may occur which can give rise
25 to post-surgical infections in the treated person; it may be difficult to administer a correct haemostatically effective amount of the compounds.

- It is also known that the administration of said blood coagulation accelerating factors may be carried out by soaking haemostatic sponges as defined above in aqueous solutions thereof prior to the ap-
30 plication of the sponges. By applying such a procedure, the haemostatic activity of the sponges will be significantly enhanced, the degree of enhancement being dependent on which compounds are used and the amounts thereof used. However, the inconveniences involved in the
35 administration of aqueous solutions as described above are substan-

tially the same even when haemostatic sponges are used simply as a physical vehicle.

These inconveniences can be appropriately overcome by the preparation of haemostatic sponges to which the said blood coagulation factors have been added prior to packaging, the preparation process being carried out in such a way that sterile conditions in the thus obtained ready-to-use blood coagulation factor-containing haemostatic sponges are secured. In the preparation of such haemostatic sponges the process by which the coagulation factors are added to the spongy material must be selected so as to maintain the physical structure and the mechanical strength of the material. Furthermore, the selected process must be cost-competitive and should preferably not comprise heavily energy-consuming steps such as freeze-drying, a further disadvantage of which being that the resulting freeze-dried material tends to become fragile to an extent which makes the structural integrity of the said material difficult to maintain during handling of the finished product including surgical application.

US 2,558,395 discloses a ready-to-use undenatured gelatine haemostatic sponge containing thrombin, the preparation of which comprises freeze-drying of a foam of an aqueous solution containing undenatured water-soluble gelatine and thrombin.

US 4,265,233 discloses wound healing material comprising a structure made from compounds such as absorbable gelatine, collagen, polyglycolic acid and polylactic acid to which blood coagulation factors have been fixed by covalent bonding, the preparation of which involves dipping the preformed structure in an aqueous solution of the coagulation factors and subsequent freeze-drying for 20 hours.

DK Patent Application No. 398/88 discloses a wound dressing comprising a stable thrombin composition and a substrate such as haemostatic sponges comprising a porous structure of collagen or denatured gelatine onto which the said composition is absorbed by saturating said substrate with a thrombin solution stabilized with glycerol and a phosphate- or citrate buffer, the saturated sponges subsequently being freeze-dried.

According to the known art, ready-to-use blood coagulation factor-containing dry haemostatic sponges are prepared either by forming a foam of undenatured gelatine which is thereafter freeze-dried or by saturating a preformed dried sponge with a solution of the blood coagulation factor, the wet sponge thereafter being freeze-dried. The former preparation technique involves the disadvantage that the sponge material can not be denatured thermally or chemically, such treatments being destructive for blood coagulation factors. The latter technique implies the possibility to apply denatured water-insoluble sponge materials which as explained previously is advantageous, such sponges retaining their physical structure after application to the site of bleeding for considerably longer time than undenatured sponges. However, said technique implies two subsequent drying processes, of which at least one comprises a time- and heavily energy-consuming freeze-drying process. This secondary drying process will add considerably to the production costs. Another disadvantageous aspect of the said technique is that that the physical structure of the sponge may be damaged or weakened during remoistening, freeze-drying, packaging, and further handling.

20 SUMMARY OF THE INVENTION

The present inventors have now found that a stabilized thrombin solution can be prepared in which the thrombin activity is substantially retained during storage at room temperature for at least 72 hours; during injection into a dry biologically absorbable, haemostatic sponge; during air-drying of the thus injected sponge at a temperature in the range of 30 - 100°C for at least 20 - 24 hours; during packaging and ionizing irradiation; and furthermore during storage of the thus prepared ready-to-use dry blood coagulation factor-containing biologically absorbable haemostatic sponge at room temperature or higher temperatures for several months.

Accordingly, the present invention relates to a haemostatic sponge comprising a porous structure of biologically absorbable solid material, such as denatured gelatine, a haemostatically effective amount

of thrombin, and one or more thrombin-stabilizing agents, said sponge having a total water content of below 50% by weight, and the thrombin-stabilizing agents being selected from naturally occurring amino acids, mono- or disaccharides, polyglycols, proteins, and mixtures thereof. In a further aspect of the invention, the said haemostatic sponge may further contain one or more buffering salts and mixtures thereof; polyvalent alcohols; sodium chloride; haemostatically effective amounts of one or more blood coagulation factors other than thrombin; an anti-fibrinolytic agent; or one or more antimicrobial agents.

The present invention further relates to a method for preparing a haemostatic sponge as defined above, which method comprises the following steps: 1) preparing a sponge comprising a porous structure of biologically absorbable, solid material, such as denatured gelatine; 2) introducing into said sponge by injection at a multiplicity of sites an aqueous solution of thrombin, one or more thrombin-stabilizing agents and optionally one or more buffering salts; and optionally a polyvalent alcohol; and optionally sodium chloride; and optionally further blood coagulation factors; and optionally one or more anti-fibrinolytic agents; and optionally one or more antimicrobial agents; 3) air-drying the sponge from step 2) at a temperature in the range of 30 - 100°C for a time period sufficient to reduce the water content to below 50%.

One aspect of the method is that the above injected stabilized thrombin solution has physical characteristics ensuring that the structure of the sponge being injected is substantially preserved during the injection said physical characteristics including viscosity and surface tension which are selected so as to facilitate the injection without resulting in leakage of the injected liquid to the surfaces of the sponge material. A further aspect relating to the surgical applicability of the haemostatic sponges of the present invention is that the original shape of the sponge material subsequent to the injection of the stabilized thrombin solution may be modified by compression or by a treatment resulting in an expansion of the sponge. In a still further aspect of the method packaged sponges are sterilized by ionizing irradiation.

The present invention further relates to the use of a haemostatic sponge according to the invention as a haemostatic adjunct in medical, veterinary, or dental surgery and furthermore to the use of a haemostatic sponge according to the invention for the preparation of a haemostatic adjunct to be used in medical, veterinary, and dental surgery.

DETAILED DISCLOSURE OF THE INVENTION

The general effect of haemostatic sponges is to enhance the physiological blood coagulation process thereby reducing the time elapsing from opening of the blood vessels until a firm blood clot has been formed. This period is generally referred to as "the blood coagulation time". In this context, the term "haemostatic" should be understood to mean the effect of an object or an agent which reduces the blood coagulation time, thereby promoting haemostasis.

The present invention relates to a dry haemostatic sponge comprising a porous structure of biologically absorbable, solid material, such as denatured gelatine containing a haemostatically effective amount of thrombin and one or more thrombin-stabilizing agents. The denaturation of a gelatine sponge structure is preferably brought about by heating said structure in air at a temperature in the range of 100 - 160°C, preferably at a temperature of 150°C for 0.5 - 4 hours. Proteins such as gelatine may also be denatured by chemical treatments with acids, bases, solvents, aldehydes, urea, or detergents such as sodium dodecyl sulphate and guanidine hydrochloride. By the said denaturation the chemical characteristics of the gelatine molecule is modified, which modification results in loss of water solubility. Furthermore, the gelatine is hardened, which is considered advantageous in relation to the use of the sponge as a haemostat, the mechanical strength of the structure being greatly increased as compared to a non-denatured gelatine structure which when becoming moistened will become dissolved and thereby collapse. In contrast, a hardened, denatured gelatine sponge will retain its structure for a considerable period of time after application to a bleeding site. Denatured

gelatine can be degraded by proteolytic tissue enzymes to absorbable smaller molecules, whereby the denatured gelatine sponge when applied in tissues is absorbed within about 3 weeks and when applied on bleeding surfaces and mucous membranes within 3 - 5 days.

5 Even though a denatured gelatine sponge as defined above represents a particularly suitable embodiment of the present invention, it is obvious to the person skilled in the field that other biodegradable compounds currently used for haemostatic purposes, such as collagen, chitin, cellulose, polyglycolic acid, and polylactic acid, said
10 compounds being in their native form or structurally modified, may also be used without being regarded as departures from the spirit and scope of the invention. Examples of said currently used haemostatic sponges may be found e.g. in Physicians' Desk Reference, 1988, Medical Economics Company Inc., Oradell, N.J., U.S.A.

15 As it has been explained hereinbefore prothrombin which is a precursor for thrombin consists of two fragments, one of which is the active thrombin. Prothrombin is activated to thrombin by the removal of the amino-terminal fragment, brought about in the presence of calcium ions, by a complex of factor Xa and two cofactors, factor Va
20 and phospholipids, both of which are present on the surface of activated platelets. Besides occurring on the surface of activated platelets, prothrombin activator substances are found in lung extracts. In the present context, the term "thrombin" is understood to include the precursor therefor which precursor as it is explained
25 above comprises the active thrombin. It is contemplated that prothrombin in a dried haemostatic sponge according to the invention will become activated to active thrombin in the presence at the site of bleeding of the abovementioned cofactors. When haemostatic sponges according to the invention are impregnated with prothrombin it may be
30 advantageous to include at least one prothrombin activator selected from the group consisting of factor Xa, factor Va, procoagulant phospholipids and calcium ions.

According to the invention the haemostatic sponge contains a haemostatically effective amount of thrombin, the average content of which
35 is in the range of 0.1 - 300 NIH units per cm³, preferably 0.2 - 60

NIH units per cm^3 , and in particular 1 - 40 NIH units per cm^3 . Thrombin preparations may vary in their fibrinogen-clotting activity depending on the purity and the concentration. A haemostatically effective amount of thrombin therefore relates to a particular fibrinogen-clotting activity which is determined by a procedure as described in details in Example 1 according to which the activity of a sample or a thrombin preparation with an unknown thrombin activity after appropriate dilution is compared with the activity of a standard thrombin preparation. The term "NIH (National Institute of Health) unit" refers to a thrombin activity contained in a specified weight of a NIH standard thrombin preparation, which was also used by the present inventors.

It is contemplated that the incorporation of an effective fibrinogen-clotting amount of thrombin in a sponge which *per se* has a haemostatic effect will increase said effect by further accelerating the activation of platelets and also directly by initiating the conversion of fibrinogen to fibrin immediately after rehydration of the thrombin which takes place almost momentarily in the sponge structure, said structure being able to absorb 50 times its weight of liquid. It is furthermore contemplated that an increase of haemostatic effect can be similarly obtained by adding to a thrombin-containing haemostatic sponge haemostatically effective amounts of other blood coagulation factors involved in the formation of the prothrombin activation system as defined above, such as the factors VIIa, VIIIa, IXa, XIa, and XIIa. In a still further aspect of the invention the factor XIIIa may be added, the incorporation of which will bring about an acceleration of the formation of a firm clot resistant to dissolution.

Thrombin is an unstable biological compound, which in the present context is understood to mean a compound which upon storage in an aqueous solution at room temperature or above room temperature or when it is exposed to temperatures above room temperature, such as temperatures in the range of 30 - 60°C in solution, in partially dried form, or in dried form loses its activity at a rate depending on the temperature levels and the period of time. The activity loss may be due to autolysis (self-degradation) or to denaturation. Conse-

quently, commercial preparations of thrombin are distributed as frozen solutions or in the freeze-dried form. It is known that aqueous solutions of thrombin can be prepared which in comparison with purely aqueous solutions have an increased room temperature stability by the addition of one or more thrombin-stabilizing agents.

It has now surprisingly been found by the present inventors that it is possible to prepare thrombin solutions comprising one or more stabilizing agents which are not only resistant to thrombin activity losses during storage at room temperature of aqueous solutions, but which can also preserve thrombin activity during injection of said stabilized solutions into dry, biologically absorbable, haemostatic sponges, and furthermore during subsequent drying of the thus injected sponges in air at temperatures in the range of 30 - 100°C for a time period of 0.5 - 4 hours, and even for 24 hours as described in details in the Examples below, and finally during packaging, ionizing irradiation, and storage of the packaged thrombin-containing sponges for several months at room temperature or above, and additionally having physical characteristics substantially securing that the structure of the sponge material is not changed during injection. The stabilizing agents may be selected from naturally occurring amino acids, mono- or disaccharides, polyvalent alcohols, polyglycols, proteins, and mixtures thereof.

By naturally occurring amino acids is understood any amino acid which is found in biologically produced proteins, including essential and nonessential dietary amino acids in their two stereoisomeric forms: arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, alanine, aspartate, cysteine, glutamate, glycine, proline, serine, tyrosine, glutamine, and asparagine. According to the present invention, preferred amino acids are: glycine, lysine and arginine.

Suitable monosaccharides in the context of the invention may be selected from D- or L-forms of pentoses, such as ribose, arabinose, xylose, and lyxose and hexoses such as allose, altrose, glucose, mannose, gulose, idose, galactose, talose and derivatives thereof, e. g. pentosamines, hexosamines and glucuronic acid. Disaccharides may

be selected from lactose, saccharose, maltose, fructose, and cel-
lubiose, including derivatives thereof.

In a preferred embodiment of the sponge of the invention, a poly-
valent alcohol is used as a further thrombin-stabilizing agent. A
5 suitable polyvalent alcohol may be selected from ethylene glycol,
diethylene glycol, propylene glycol, glycerol, mannitol, inositol,
xylitol, erythritol, pentaerythritol, pentitols, hexitols, such as
sorbitol, and heptitols. Furthermore, polyglycols, such as polypro-
pylene glycol and polyethylene glycols may be useful as thrombin-
10 stabilizing agents. Among the latter group of compounds polyethylene
glycols having a molecular weight in the range of 400 - 20,000 are
preferred, such as 6,000.

Proteins which are not enzymes may be suitable as stabilizing agents
for thrombin in aqueous solution and during drying of the injected
15 haemostatic sponge. Thrombin-stabilizing proteins may be selected
from serum albumin, egg albumin, gelatine, collagen, casein, keratin,
and globulins.

The total amount of thrombin-stabilizing agents incorporated in the
20 haemostatic sponge may vary within relatively wide limits, inter
alia dependent on the particular composition of a mixture of the
above stabilizing agents and the nature and concentration of the
selected thrombin preparation. Thus, an adequate amount of thrombin-
stabilizing agents may be in the range of 0.05 - 0.5 mg per NIH unit
25 of thrombin.

In one particular embodiment of the invention, the haemostatic sponge
having a water content below 50%, comprising thrombin and thrombin-
stabilizing agents further contains one or more buffering salts to
obtain a pH in the range of 5.00 - 7.00 in the thrombin solution
30 prior to injection into the sponges, during the subsequent drying
process and after rehydration. The buffering salts being added to the
injected thrombin solution are selected from alkaline metal salts,
such as acetates, citrates, phosphates, hydrogen phosphates, car-
bonates, hydrogen carbonates, and succinates. Other useful buffering
35 compounds may be selected from imidazole, TRIS, and zwitteranionic

buffering systems. It may also be useful to prepare mixtures of the above buffering salts.

In a further aspect of the invention the haemostatic sponge may contain sodium chloride in the range of 0.0006 - 1.8 mg per NIH unit of thrombin, preferably in the range of 0.003 - 1.0 mg per NIH unit of thrombin, and in particular 0.005 - 0.2 mg per NIH unit of thrombin, the sodium chloride being added to the thrombin solution. The incorporation of sodium chloride in the haemostatic sponge will aid in the maintenance of isotonic conditions within the sponge after application to the bleeding site.

It may be advantageous to enhance further the haemostatic effect of the thrombin-containing haemostatic sponge by incorporating in said sponge blood coagulation factors other than thrombin in haemostatically effective amounts, said blood other blood coagulation factors being added to the thrombin solution. Such additional blood coagulation factors may be incorporated as single compounds or in mixtures and may be selected from the factors involved in the formation of the prothrombin activation system, e. g. the factors Va, VIIa, VIIIa, IXa, Xa, XIa, XIIa, and calcium ions. Furthermore factor XIIIa may be added, said factor having an effect on the formation of a firm clot which is resistant to dissolution.

The mammalian body has an innate fibrinolytic system which is activated by deposition of fibrin. By dissolving fibrin, this system helps keep open the lumen of an injured blood vessel. However, in a situation where rapid haemostasis is aimed at, the said fibrinolytic activity may counteract the haemostatic effect of a haemostatic adjunct, such as a haemostatic sponge according to the present invention. The fibrinolytic system involves the activation of plasminogen, a plasma precursor for an active proteolytic enzyme, plasmin, which is bound to lysine residues on the fibrin. Accordingly, it may be advantageous to have agents incorporated in the haemostatic sponge of this invention, which has an anti-fibrinolytic effect, e. g. selected from tranexamic acid, ϵ -aminocaproic acid, aprotinin, pepstatin, leupeptin, antipain, chymostatin, and gabexate mesylate in anti-

fibrinolytically effective amounts, said agents being added to the thrombin solution.

In a still further aspect of the present invention one or more antimicrobial agents are incorporated in the haemostatic sponge, said agents being selected from bactericidal or bacteriostatic agents, such as antibiotics and sulphonamides, antiviral compounds, antimycotic agents and anti-infectives. The choice of suitable antimicrobial agents in the context of the invention will depend on several factors, such as the anatomical region where the haemostatic sponge is applied, the microflora composition being different from one habitat to the other and the severity and extent of the bleeding to be arrested. Antibiotics may be selected from e. g. β -lactams, penicillins, cephalosporins, monobactams, macrolides, polymyxins, tetracyclines, chloramphenicol, thrimethoprim, aminoglycosides, clindamycin, and metronidazole; sulphonamides may as an example be selected from sulphadimidine or sulphadimethoxin; antimycotic agents may be selected from amphotericin B, ketoconazol, and miconazol; and antiviral agents from idoxuridine and azidothymidin. Suitable anti-infectives may as an example be selected from halogens, chlorohexidine, and quarternary ammonium compounds. The incorporation of said antimicrobial agents is carried out by adding such agents to the thrombin solution.

It is contemplated that a low content of residual water in the haemostatic sponge of this invention is advantageous in respect of preserving the activity of the thrombin and optionally further biological compounds. The extent to which the injected aqueous solvent can be removed by the post-injection drying process depends on a number of factors, including the water content prior to drying, the composition and the osmolarity of the injected solution, the drying temperature, the drying period, and the porous structure of the sponge. According to the invention, the total water content is at least below 50%, but preferably below 30%, more preferably below 20%, in particular below 15%, more particularly below 10%, and particularly preferred below around 7%.

The present invention further relates to a method for preparing a haemostatic sponge as described above, the method comprising the following steps:

- 1) preparing a sponge comprising a porous structure of biologically absorbable, solid material;
- 2) injection into said sponge at a multiplicity of sites an aqueous solution of thrombin, one or more thrombin-stabilizing agents and optionally one or more buffering salts; and optionally sodium chloride; and optionally further blood coagulation factors; and optionally one or more anti-fibrinolytic agents; and optionally one or more antimicrobial agents; and
- 3) air-drying the sponge from step 2) at a temperature in the range of 30 - 100°C, preferably 40 - 50°C, such as 50°C, for a time period sufficient to reduce the total water content to below 50%, preferably below 30%, more preferably below 20%, in particular below 15%, more particularly below 10% and particularly preferred below around 7% by weight. The haemostatic sponge into which the above thrombin solution is injected is prepared in accordance with the above description. In this context the term "sponge" is understood to mean any suitable size and formate of any haemostatically suitable biodegradable material, such as absorbable gelatine. It may thus be convenient to prepare rather large plates of the sponge material having a suitable thickness, e. g. 5 - 20 mm, which then subsequently to injection of the stabilized thrombin solution are cut into appropriately sized sponge units.

In this context the thrombin-stabilizing agent or agents may be selected from naturally occurring amino acids, mono- or disaccharides, polyglycols, proteins, polyvalent alcohols, or mixtures thereof.

The stabilized thrombin-containing solution should preferably be prepared just before the injection into the sponge. It may however be prepared a few days in advance and stored until use, the storage period depending on the storage temperature. The concentration of thrombin in the said solution is in the range 500 - 2000 NIH units per ml. The source of thrombin for preparing the solution can be any

suitable commercial preparation of purified thrombin having a sufficiently high content of thrombin activity, such preparations having been rendered stable by freeze-drying or by being kept as a frozen solution. Prior to use such preparations are reconstituted or thawed. Furthermore the source of thrombin may be preparations of prothrombin in which the prothrombin can be activated by the addition of prothrombin activating substances as defined hereinbefore. In commercial procedures for the activation of prothrombin to thrombin, purified lung extract is commonly used as the source of activator substances.

The thrombin-stabilizing agents are added to the solution in any appropriate order. The concentration of amino acids is in the range 0.01 - 0.1M, a preferred concentration being 0.05M. Mono- or disaccharides are added at a concentration of 0.01 - 0.1M, 0.05M being a preferred concentration. Polyglycols may be added at a concentration varying between 0 and 50% by volume, e. g. 20%. A preferred polyglycol is polyethylene glycol having a molecular weight in the range of 400 - 20,000, a preferred molecular weight being 6,000. When proteins are included as a stabilizing agent the concentration thereof is suitably in the range of 0.1 - 5% by weight, a preferred concentration being 3% by weight. Any thrombin-stabilizing protein may be applied according to the invention, although albumin is a preferred protein. According to the invention, the concentration of polyvalent alcohols should be in the range of 0.01 - 0.1M, preferably 0.05M.

The total concentration of thrombin-stabilizing agents in the thrombin solution for injection should be in the range of 7 mg - 640 mg per ml, preferably in the range of 150 - 400 mg per ml.

Optionally, the thrombin solution may contain sodium chloride within a concentration range of 0.5 - 1.5%, the concentration preferably being 0.9%. Additionally, one or more buffering salts may be added to the solution in order to bring about a pH in the range of 5.0 - 7.0. The concentration of buffering salts is in the range of 0.001 - 0.1M, a preferred concentration being 0.01M.

In a particular aspect of the method, the physical characteristics of the injected stabilized thrombin solution is selected so as to secure an appropriate "injectability" of said solution which term is understood to mean that a suitable quantity of the solution can be delivered per unit of time without the use of extraordinary forces and substantially without any leakage of the injected liquid to the surfaces of the sponge material. A further aspect of the physical characteristics of the solution is that said characteristics are adapted in such a way that substantially no deformation of the sponge structure occurs. A more detailed description of these physical requirements of the injected thrombin solutions is given in Example 5 below. In relation to defining said appropriate physical characteristics, it has been found that part of the aqueous solvent advantageously is replaced by a monovalent alcohol, such as methanol, ethanol, propanols, or butanols, the concentration of said alcohol being in the range of 0.1 - 40% by volume.

According to the invention the method for preparing a haemostatic sponge implies the injection into the dry sponge material of a stabilized thrombin solution carried out by means of a single or preferably a multiple injection device delivering appropriately preset quantities of said solution into the sponge, said quantities preferably being in the range of 0.01 - 0.5 ml, most preferably 0.03 ml. The injection needles applied in the injection device has a diameter so that the sponge structure is not damaged to an extent which in any way might affect the visual appearance of the final haemostatic product. A further aspect of the injection method is that the injection needle points are placed on the same or staggered planes at intervals of 5 - 20 mm, preferably 8 - 12 mm.

In a particular embodiment of the invention, the drying of the injected sponge as referred to above may also be carried out at air pressures below atmospheric pressures, the reduced air pressure being e.g. in the range of 0.2-400 mm Hg, whereby a lowering of the water content to the same levels as defined above can be achieved within the same period of time but in a lower temperature range. Drying of the injected sponges at lower temperatures may be advantageous in regard to preservation of thrombin stability.

In a still further aspect of the method, the original shape of the sponge material may be modified subsequent to injection by compression or rolling or by extrusion under reduced pressure. In the former case sheets are formed, the thickness of which depends on the compression force applied. Such sheets may be particularly useful as haemostatic adjuncts on open body surfaces or on mucosal membranes, whereas extruded haemostatic sponges according to the invention may be particularly suitable in body cavities. After injection of thrombin solution, drying, and optionally modification of shape, the sponge material may be cut into surgically suitable sizes haemostatic sponge units.

Subsequent to appropriate packaging, the ready-to-use dry thrombin-containing haemostatic sponges are optionally sterilized by ionizing irradiation, i. e. β - or γ -irradiation, the effective dosage being in the range of 10 - 50 kGy, preferably in the range of 20 - 40 kGy.

The present invention further relates to the use of a haemostatic sponge according to the invention as a haemostatic adjunct in medical, veterinary, and dental surgery. The present invention furthermore relates to the use of a haemostatic sponge according to the invention for the preparation of a haemostatic adjunct to be used in medical, veterinary, and dental surgery.

The invention also relates to a method for arresting bleeding comprising the application to the site of bleeding of a haemostatic sponge according to the invention.

The following examples serve to illustrate the invention in further detail, but are not intended to limit the invention in any way.

EXAMPLES

Example 1

Preservation of thrombin activity in stabilized aqueous solutions stored under different temperature conditions and after injection of the solutions into Spongostan® standard sponges before and after drying

Experiment 1

A stabilized thrombin solution was prepared which solution contained the following ingredients:

10	L-lysine	457 mg
	Lactose	900 mg
	PEG 6000 1)	10 g
	NaCl	400 mg
	Thrombin solution 7802	2 ml
15	Water, ad	50 ml

1) Polyethylene glycol

The thrombin was in the form of a frozen, buffered solution with a concentration of about 3500 NIH units per ml obtained from Parke-Davis. (Division of Warner-Lambert).

20 The thrombin activity of the solution was measured immediately after the preparation and after storage at different temperatures and periods of time according to the following method:

Measurement of thrombin activity

Principle:

25 Fibrinogen in standardized plasma was used as the substrate for thrombin. The coagulation time for a sample was compared with the coagulation time for two or more standard solutions of thrombin.

Apparatus

- Coagulometer KC 4A, Amelung GmbH
Fine steel balls, Amelung GmbH, Item No. Z03100
Combitips 2.5 ml, Eppendorf, Item No. 0030048.016
5 Cuvettes, Amelung GmbH, Item No. 834011

Reagents

- Imidazole, Riedel de Haen, Art. No. 33314
Sodium chloride, Merck Art. No. 6404
Hydrochloric acid dilute, Ph. Eur. 2nd. Ed., VII.1.1.
10 Control Plasma Normal, Nycomed, Item No. 207001
Polyethylene glycol 6000, Merck Art. No. 807491.
Thrombin, Parke-Davis N0071-4173-35
Water, Milli Q quality
- Imidazole buffer, 17.2 g imidazole was dissolved in 900 ml 0.1 N HCL.
15 pH was adjusted to 7.25 with 10 % HCl and the solution was diluted to 1000 ml with water.
- PEG-6000 buffer, 9.00 g sodium chloride and 5.00 g polyethylene glycol were dissolved in 300 ml of water. 58.8 ml imidazole buffer was added and the solution was diluted to 1000 ml with water.
- 20 Plasma: 1.00 ml water was added to each vial of Control Plasma Normal and the solution was mixed. If more than one vial was used, all vials were mixed to avoid variation. The plasma was frozen at -20°C.

Before use, frozen plasma was melted and stored in an ice bath.
Plasma was not allowed to be frozen twice.

25 Standards

The thrombin was dissolved in an isotonic sodium chloride solution and diluted to 100 NIH/ml with PEG-imidazole buffer and frozen in portions of 3 ml in polypropylene tubes.

Working standards: A standard tube was melted and diluted to 0.3-0.5 NIH/ml with PEG-imidazole buffer.

- Test solution: The sponge was soaked in 30-40 ml PEG-imidazole buffer and the solution was transferred to a suitable volumetric flask. The
 5 extraction procedure was carried out twice. The combined solutions with PEG-imidazole buffer were diluted to volume. In some cases, further dilutions were necessary to achieve a final concentration of 0.4 NIH/ml.

Method

- 10 0.6 ml standard or test solution was preheated for 3 minutes in a cuvette. 100 μ l plasma was pipetted into a cuvette. After having added a steel ball, the cuvette was placed in the measuring position of the coagulometer. After 3 minutes, 100 μ l standard or test solution was added to the plasma cuvettes with the automatic pipette.
 15 The automatic pipette activated the timer of the coagulometer and the coagulation time of the sample was measured.

The results of the activity measurements of the aqueous solutions after storage are summarized in Table 1:

Table 1

- 20 Thrombin activity (NIH units/ml) in a stabilized thrombin solution after storage

	After Storage				
	Initial	2 h. at 40°C	2 h. at 60°C	72 h. at RT ¹⁾	72 h. at -15°C
25	140	180	0	120	170

1) Room temperature

0.7 ml of the above solution was injected into Spongostan® Standard sponges having a dimension of 70 x 50 x 10 mm (Ferrosan, Copenhagen, Denmark) which were then dried at room temperature (RT) (20-25°C) and 50°C, respectively for 24 hours. The thrombin activity was measured and the results obtained are found in Table 2:

Table 2

Thrombin activity in Spongostan® sponges before and after drying (NIH units/sponge)

Wet sponge after injection	Sponge dried at 50°C/24 h.	Sponge dried at RT/24 h.
100	58	58

Experiments 2-16

A further 15 stabilized thrombin solutions were prepared the compositions of which are described in Table 3. Thrombin activities of these solutions after storage under the conditions described in Experiment 1 were measured as described in Experiment 1, and the results are summarized in Table 4. Furthermore, the solutions were injected into Spongostan® sponges which were dried as described in Experiment 1, and the thrombin activities were measured after drying according to the method specified in Experiment 1. The results of the measurements of thrombin activities in dried sponges are shown in Table 5.

Table 3a

Compositions of stabilized thrombin solutions, Nos. 2-9 used in Experiments 2-9

5	Thrombin solution Nos.					
	Compounds	Unit	2	3	4	5
	Lysine	mg			457	457
10	Arginine	mg	436			
	Glycine	mg		188		
	Lactose	mg				
	Glucose	mg	450		450	450
15	Sorbitol	mg		450		
	PEG 6000	g	10	10	10	10
	NaCl	mg	400	400	400	400
	Thrombin sol. 7802	ml	2	2	2	2
20	Water, ad	ml	50	50	50	50

Table 3a continued:

		Thrombin solution Nos.				
	Compounds	Unit	6	7	8	9
5	Lysine	mg				
	Arginine	mg		436	436	436
10	Glycine	mg	188			
	Lactose	mg		900		
	Glucose	mg	450			
	Sorbitol	mg			450	
	PEG 6000	g	10	10	10	10
15	NaCl	mg	400	400	400	400
	Thrombin sol. 7802	ml	2	2	2	2
	Water, ad	ml	50	50	50	50

Table 3b

Compositions of stabilized thrombin solutions, Nos. 10-16, used in Experiments 10-16

5	Thrombin solution Nos.					
	Compounds	Unit	10	11	12	13
	Lysine	mg	457			
10	Arginine	mg				
	Glycine	mg		188	188	
	Lactose	mg			900	900
	Glucose	mg				
15	Sorbitol	mg	450			
	PEG 6000	g	10	10	10	10
	NaCl	mg	400	400	400	400
	Thrombin sol. 7802	ml	2	2	2	2
20	Water, ad	ml	50	50	50	50

Table 3 b continued:

	Compounds	Unit	Thrombin solution Nos.		
			14	15	16
5	Lysine	mg			
	Arginine	mg			
10	Glycine	mg			
	Lactose	mg			
	Glucose	mg	450		
	Sorbitol	mg		450	
	PEG 6000	g	10	10	10
15	NaCl	mg	400	400	400
	Thrombin sol. 7802	ml	2	2	2
	Water, ad	ml	50	50	50

Table 4

Thrombin activities (NIH/ml) in stabilized thrombin solutions Nos. 2-16 after storage

5	Thrombin solution No.	Initial	After storage			
			2h/40°C	2h/60°C	72h/RT	72h/-15°C
10	2	170	0	0	75	170
	3	180	200	0	140	180
	4	220	200	0	150	180
	5	210	200	0	150	160
	6	170	180	0	135	150
15	7	170	0	0	75	110
	8	170	0	0	70	130
	9	170	0	0	50	120
	10	190	200	0	110	140
	11	140	140	0	110	110
20	12	140	140	0	110	120
	13	150	130	0	110	120
	14	140	140	0	100	110
	15	140	140	0	110	120
	16	140	150	0	110	110

Table 5

Thrombin activities injected in Spongostan® sponges before and after drying (NIH units/ml)

5	Injected thrombin sol.No.	Activity in		
		wet sponges after inject.	sponges dried at 50°C/24 h.	sponges dried at RT/24 h.
10	2	90	26	38
	3	90	59	51
	4	100	59	61
	5	100	27	61
	6	90	57	55
15	7	90	42	57
	8	80	58	50
	9	90	51	53
	10	120	58	59
	11	70	63	55
20	12	70	63	54
	13	80	58	60
	14	80	57	45
	15	80	58	43
	16	80	58	35
25				

Conclusions

In the thrombin solutions containing stabilizing agents, a rather high recovery of thrombin activity was demonstrated in all of the test solutions after storage for 24 hours at room temperature and at -15°C. None of the solutions resisted storage at 60°C for 2 hours whereas recoveries of thrombin activity after storage at 40°C for 2

hours were high in a majority of the solutions (about 100%) except the solution Nos. 2, 7, 8, 9 from which no activity at all could be recovered. These results clearly indicate that the composition of the stabilizing agents is significant and that it is possible to stabilize a thrombin solution by the addition of suitable stabilizing agents. It has furthermore been demonstrated that the stabilized thrombin solutions when injected into denatured gelatine sponges retain their blood coagulation activities to a considerable degree after drying of the injected sponges for 24 hours at 50°C and room temperature, respectively.

EXAMPLE 2

Recovery of thrombin from Spongostan® Standard sponges after injection of a thrombin solution and subsequent drying at 50°C for 20 hours

A stabilized thrombin solution (solution V) was prepared containing the following ingredients:

Thrombin (Hoffmann La-Roche, freeze-dried)	30,000 units
L-lysine	230.4 mg
Glycerol	9 g
PEG 6000	4 g
NaCl	270 mg
Water, ad	30 ml

The freeze-dried thrombin was very difficult to dissolve so the final solution contained 226 units/ml determined according to the method described in Example 1.

0.7 ml (158 units) of this solution was injected into Spongostan® Standard sponges and dried at 50°C for 20 hours.

Results

Thrombin activity in wet sponge: 130 units/sponge

Thrombin activity in any dried sponge: 0 units/sponge

Conclusion

- 5 With the above formulation of the thrombin solution comprising glycerol, it was not possible to demonstrate recovery of thrombin activity after drying of the sponges at 50°C for 20 hours.

EXAMPLE 3

- 10 *Recovery of thrombin activity in dried Spongostan® Standard sponges after storage in paper bags and aluminium foil bags at different temperatures and after β -irradiation*

A stabilized thrombin solution (solution 12) was prepared which solution contained the following ingredients:

	Thrombin (Hoffmann La-Roche, freeze-dried)	30,000 units
15	L-glycine	112.8 mg
	Lactose	540 mg
	PEG 6000	4 g
	Sodium chloride	270 mg
	Water, ad	30 ml

- 20 The freeze-dried thrombin was difficult to dissolve so the final solution as defined above contained only 59 units/ml as determined by the method described in Example 1. 0.7 ml (41 units) of the solution was injected into Spongostan® Standard sponges which were then dried at 50°C for 20 hours. The dried sponges were packaged in paper bags
25 and aluminium foil bags, respectively, and subsequently stored for 2 weeks at 20°C, 40°C and 60°C, respectively.

In addition, some of the sponges were sterilized by β -irradiation at 25 kGy or 35 kGy.

Results

Thrombin activities were measured in the non-irradiated as well as in the irradiated sponges. The results are shown in Tables 6 and 7 below:

5 Table 6

Recovery of thrombin activities in dried, packaged sponges after storage

10

Thrombin activity,
% of the activity of the injected
solutions after storage at:

15

Sponges in	Ini- tial	20°C		40°C		60°C	
		1 week	2 weeks	1 week	2 weeks	1 week	2 weeks
Paper bags	90%	93%	86%	96%	110%	80%	24%
Aluminium foil bags	90%	95%	85%	91%	84%	0%	0%

20 Table 7

Recovery of thrombin activities in dried, irradiated sponges into which a stabilized thrombin solution had been injected

25

Thrombin activity, % of activity
in the injected solution

Aluminium foil bags		Paper bags	
25 kGy	35 kGy	25 kGy	35 kGy
70%	55%	68%	55%

30

Conclusions

When injected into denatured gelatine Spongostan® sponges, the present stabilized thrombin solution showed a 90% recovery immediately after drying of the injected sponges, without any significant losses during storage at 20°C and 40°C for 2 weeks. Even at 60°C the sponges packaged in paper bags had retained 80% of the initial activity of the injected solution after 1 week, and still after 2 weeks, 24% activity could be recovered. At 60°C, recovery was poor when sponges were packaged in aluminium foil bags.

- 10 Thrombin activity in the dried sponges was not effected to a significant extent by the applied β -irradiations.

EXAMPLE 4

Recovery of thrombin activity in sponges injected with a thrombin solution containing an acetate buffer and glycerol

- 15 A stabilized thrombin solution (solution A) was prepared which solution contained the following ingredients:

Thrombin	15,625 NIH units
L-lysine	460.8 mg
Glycerol (85%)	18 ml
20 PEG 6000	12 g
Acetate buffer, ad	60 ml

- A frozen thrombin preparation from Warner-Lambert Company, Morris Plains, N.J., U.S.A. was used. After thawing, the preparation was dialyzed in an acetate buffer containing 0.01 M acetate and 0.09% NaCl. The PEG was dissolved in the acetate buffer together with the L-lysine. When the glycerol was added, a precipitation occurred. It was only possible to dissolve the precipitate by heating to 38°C-40°C. The final solution was analyzed to contain 420 NIH units of thrombin. 0.7 ml (294 units) of this solution (38°C) was injected
- 25

into Spongostan® standard sponges which was thereafter dried at 50°C for 20 hours.

Results

Thrombin activity in wet sponges: 265 units
5 Thrombin activity in dried sponges: 0 units

Conclusion

The above formulation containing glycerol and acetate buffer did not stabilize the thrombin sufficiently to resist the applied drying process.

10 EXAMPLE 5

Studies on the influence of the composition of stabilized thrombin solutions on thrombin activity recoveries during the preparation of irradiated, dried denatured gelatine sponges; on injectability quality; on sponge deformation after injection; and on residual water
15 *content in injected sponges after drying*

Experimental design

Twelve different stabilized thrombin solutions were prepared according to Table 8 shown below.

A frozen thrombin preparation from Warner-Lambert Company was used
20 which after thawing was dialyzed against an acetate buffer containing 0.01M acetate and 0.9% NaCl.

Table 8a

Compositions of stabilized thrombin solutions A-G tested in Example 5

			Solutions			
5	Compounds	Units	A	B	C	D
	L-Lysine	mg	460	384		
	L-Glycine	mg			188	
	Glycerol	mg	18			
10	Lactose	mg			900	
	Gelatine	mg				
	Ethanol, 96%	ml				
	PEG 6000	g	12	10	10	10
	Albumin, 20%	ml				
15	Thrombin	NIH units	43,750	43,750	43,750	43,750
	Acetate buffer, ad	ml	60	50	50	50

Table 8a continued:

		Solutions			
	Compounds	Units	E	F	G
5	L-Lysine	mg			
	L-Glycine	mg	188		
	Glycerol	mg			
	Lactose	mg		900	
10	Gelatine	mg			500
	Ethanol, 96%	ml			
	PEG 6000	g			
	Albumin, 20%	ml			
15	Thrombin	NIH units	43,750	43,750	43,750
	Acetate buffer, ad	ml	50	50	50

Table 8b

Compositions of stabilized thrombin solutions H-P tested in Example 5

			Solutions				
5	Compounds	Units	H	K	L	N	P
	L-Lysine	mg					
	L-Glycine	mg				188	188
	Glycerol	mg					
10	Lactose	mg				900	
	Gelatine	mg					
	Ethanol, 96%	ml			15.60		
	PEG 6000	g					10
	Albumin, 20%	ml		7.50		7.50	7.50
15	Thrombin	NIH units	43,750	43,750	43,750	43,750	43,750
	Acetate buffer, ad	ml	50	50	50	50	78

- 20 The above thrombin solutions were injected into Spongostan® Standard sponges in quantities of 0.7 and 1.3 ml, respectively, followed by drying of the injected sponges at 50°C for 3 hours and β -irradiation of a dosage of 25 kGy.

Measurement

- 25 During injection, the injectability quality was determined for each formulation and the easiness with which injection could be carried out was assessed according to a scoring system ranging from 5-0, in which "5" designates that injection was easy without any problems, including that the injection took place without the use of excessive
- 30 forces and that the exact deposition of the individual quantities was

possible, and "0" designates an injectability quality where the above conditions were not met at all.

Thrombin activities were measured according to the method in Example 1 at the following stages:

- 5 1) After preparation of the solution;
- 2) in the sponges immediately after injection;
- 3) in the sponges after drying at 50°C for 3 hours, and
- 4) in the dried sponges after β -irradiation at a dosage of 25 kGy.

After injection and drying of the sponges, they were examined for
10 deformation of the sponge structure. Deformations were characterized by a scoring system in which "0" indicates that no difference could be observed between the structure of a non-injected sponge and an injected sponge. "5" was used to describe a pronounced deformation where the surface of the sponge had fallen in and large dimples were
15 produced following the injection.

Finally, the residual water content of the sponges having been dried as indicated above was measured according to the standard method for loss of drying described in the European Pharmacopoeia.

Results

- 20 Results regarding pH-values of the thrombin solutions, injectability quality, deformation score, and residual water content of dried sponges are shown in Table 9.

In Table 10 are summarized the results of thrombin activity recoveries from the freshly prepared thrombin solution, from the sponges
25 after injection of 0.7 and 1.3 ml, respectively, from the injected sponges after drying and again after β -irradiation.

Table 9

pH-values of thrombin solutions, injectability quality, deformation score, and residual water content of dried sponges

5	Thrombin solution						
Parameters	A	B	C	D	E	F	
	pH of solution	6.20	6.10	6.10	6.30	6.10	6.00
10	Injectability quality	5	5	5	5	5	5
	Deformation score	1	0	0	2	3	5
15	Residual water, %	30	18	15.5	20	9.5	10.5

Table 9 continued:

		Thrombin solution					
20	Parameters	G	H	K	L	N	P
	pH of solution	5.80	6.00	6.80	6.50		
25	Injectability quality	1	0	0	5		
	Deformation score	4-5	4-5	1	3-4		
30	Residual water, %	9	10	7	7		

Table 10

Thrombin activities in 1) freshly prepared solutions, 2) newly injected sponges, 3) dried sponges, and 4) dried and β -irradiated sponges

		Thrombin activity added and thrombin activity measured, NIH units per ml or per sponge using solution:					
	Preparation stage	A	B	C	D	E	F
10	Activity added	729	875	875	875	875	875
	Measured in solution	420	800	775	655	723	845
15	Newly injected sponges	275	559	473	413	520	588
	Dried sponges, 0.7 ml	0	388	475	240	350	278
20	Dried sponges, 1.3 ml	0	N.D. x)	975	630	465	230
	β -irradiated sponges, 0.7 ml	0	333	375	180	263	200

x) Not done

Table 10 continued:

		Thrombin activity added and thrombin activity measured, NIH units per ml or per sponge using solution:					
5	Preparation stage	G	H	K	L	N	P
	Activity added	875	875	875	875	875	875
10	Measured in solution	800	810	713	845	N.D.	N.D.
	Newly injected sponges	533	550	480	573	500	508
15	Dried sponges, 0.7 ml	240	393	465	450	430	520
	Dried sponges, 1.3 ml	220	445	785	744	N.D.	N.D.
20	β -irradiated sponges, 0.7ml	205	255	368	378	373	425

In Table 11, the thrombin activities shown in Table 10 have been listed as percentages of the initial activities in the freshly prepared solutions.

Table 11

Thrombin activities from Table 10 as percentages of the initial activities in the freshly prepared thrombin solutions

5	Preparation stage	Thrombin activity added and thrombin activity measured, NIH units per ml or per sponge using solution:					
		A	B	C	D	E	F
10	Activity added	100	100	100	100	100	100
	Measured in solution	58	91	89	75	83	97
15	Newly injected sponges	54	91	77	68	85	96
	Dried sponges, 0.7 ml	0	63	78	39	57	46
	Dried sponges, 1.3 ml	0	N.D.	86	56	41	20
20	β -irradiated sponges	0	54	61	29	43	33

Table 11 continued:

		Thrombin activity added and thrombin activity measured, NIH units per ml or per sponge using solution:					
5	Preparation stage	G	H	K	L	N	P
	Activity added	100	100	100	100	100	100
10	Measured in solution	91	93	81	97	N.D.	N.D.
	Newly injected sponges	86	90	78	94	82	83
15	Dried sponges, 0.7 ml	39	64	75	74	70	85
	Dried sponges, 1.3 ml	19	39	69	66	N.D.	N.D.
20	β -irradiated sponges	33	42	60	62	61	69

Conclusions

The results found in Example 2 have been confirmed, viz that the incorporation of glycerol results in poor recovery of thrombin recovery. With solutions not containing glycerol, the recoveries of thrombin activity in dried, irradiated sponges were in the range of 30-70%, in relation to the activity measured in the injected thrombin solution.

EXAMPLE 6

The effect of Spongostan® sponges injected with a stabilized thrombin solution on the coagulation of a fibrinogen solution

An experiment was carried out in order to demonstrate that a haemostatic sponge injected with a stabilized solution thrombin and subse-

quently dried as defined hereinbefore has a fibrinogen clotting effect. Dried Spongostan® sponges were prepared which contained 1000 and 500 NIH units/sponge, respectively. The injected stabilized thrombin solution had the following composition:

5	Thrombin 2000 NIH units/ml	15 ml
	Glycine	300 mg
	Albumin, 20% (w/v)	6 ml
	0.01M acetate buffer, pH 7	ad 40 ml

10 A fibrinogen solution was prepared by dissolving 1 g of fibrinogen (KabiVitrum, Stockholm, Sweden) in 100 ml demineralized water. As control a Spongostan® sponge not injected with thrombin solution was used. It should be noted that the Spongostan® material *per se* as described hereinbefore does not affect the coagulation of fibrinogen for which reason the experiment with the pure Spongostan® sponge can
15 be considered as a blind experiment.

Forty ml aliquots of the fibrinogen solution were added to three 100 ml beakers which were left to stand at room temperature. To these beakers were added 1/4 Spongostan® sponges containing 1000, 500 and 0 NIH units of thrombin/sponge, respectively. Following the addition of
20 the sponge materials, the contents of the beakers were assessed for coagulation by measuring the time from the addition until the process of coagulation had advanced to a stage which allowed the beakers to be inverted without outflow of the clotted content.

Results

25 In the control beaker containing the control sponge material no clotting was observed after 24 hours, whereas in the beaker containing Spongostan® material injected with 1000 NIH units of thrombin/-sponge a firm clot had been generated after 2 minutes and in the beaker containing Spongostan® material with half of that amount of
30 thrombin the recorded coagulation time was 5 minutes. These results show that thrombin occurring in dried haemostatic sponges is in an active state as regards coagulation of fibrinogen.

EXAMPLE 7

The stability of thrombin in haemostatic sponges injected with stabilized solutions thereof and subsequently dried and stored at room temperature for 6 months

- 5 Eight batches of thrombin-containing Spongostan® sponges were prepared by injecting different stabilized solutions hereof into each batch of sponges by a multipipette technique, followed by drying the thus injected sponges in a drying oven at 50°C for 4 hours and measuring the initial thrombin activity. The dried thrombin-containing
10 sponges were stored at room temperature for 6 months and the remaining thrombin activities were measured after 2, 3, and 6 months, respectively. The stabilized thrombin solutions used for injection of the 8 different batches of haemostatic sponges had the following compositions:

15	Batch No.	Composition
	1	750 NIH units of thrombin/ml 0.05M acetate buffer, pH 4 3% (w/v) lactose 3% (w/v) L-glycine
20	2	1500 NIH units of thrombin/ml 0.05M acetate buffer, pH 7 0.1M L-arginine 0.1M L-glycine 3% (w/v) lactose
25		3% (w/v) albumin 10% (v/v) PEG 6000
	3	750 NIH units of thrombin/ml 0.05M acetate buffer, pH 7 0.1M L-arginine 3% (w/v) lactose
30		
	4	750 NIH units of thrombin/ml 0.05M acetate buffer, pH 4 0.1M L-arginine 0.1M L-glycine 10% (v/v) PEG 6000
35		
	5	1500 NIH units of thrombin/ml 0.05M acetate buffer, pH 4 0.1M L-arginine 3% (w/v) albumin

Continued:

Batch No.	Composition
6	1500 NIH units of thrombin/ml 0.05M acetate buffer, pH 7 10% (v/v) PEG 6000
5	
7	750 NIH units of thrombin/ml 0.05M acetate buffer, pH 7 0.1M L-glycine 3% (w/v) albumin
10	8
	750 NIH units of thrombin/ml 0.05M acetate buffer, pH 4 3% (w/v) lactose 3% (w/v) albumin 10% (v/v) PEG 6000
15	Each sponge was impregnated with 0.7 ml of stabilized thrombin solution. This means that sponges freshly impregnated with 1500 NIH units of thrombin contained 1050 units and sponges freshly impregnated with 750 NIH units contained 525 units.

Results

- 20 The residual thrombin activities were measured according to assay procedure described in Example 1. The results are summarized in the below Table 12.

Table 12

Stability data for Spongostan® sponges impregnated with stabilized thrombin solutions

	Batch	Added thrombin units/- sponge	Activity start units/- sponge	Activity 2 months' units/- sponge	Activity 3 months' units/- sponge	Activity 6 months' units/- sponge
5						
10	1	1050	940 (90)	1000 (95)	980 (93)	955 (91)
	2	1050	930 (89)	770 □ (73)	880 (84)	835 (80)
	3	525	515 (98)	525 (100)	495 (94)	450 (86)
	4	525	270 (51)	230 (44)	230 (44)	250 (48)
	5	1050	955 (91)	1010 (96)	1010 (96)	1055 (100)
15	6	1050	835 (80)	615 (59)	770 (73)	525 (50)
	7	525	490 (93)	495 (94)	475 (90)	450 (86)
	8	525	480 (91)	530 (100)	500 (95)	430 (82)

□ Large standard deviation between single measurements.

20 Each figure represents at least a double determination.

Bracketed figures indicate percent residual activity relative to the initial activity.

It appears from these results that thrombin when occurring in stabilizing solutions as defined above retains a significant proportion of the initial activity after injection into haemostatic sponges which are dried and subsequently stored at room temperature. In a majority of the tested batches of thrombin-impregnated sponges more than 80% of the activity present in the freshly injected sponge material was retained. It was also demonstrated that in most batches the loss of activity during the injection and drying process was only about 10%.

EXAMPLE 8

Stability of thrombin injected into haemostatic sponges being dried at different temperatures

A thrombin solution was prepared from a batch of frozen prothrombin preparation which was activated to thrombin after thawing by the addition of purified lung extract (PLE) in the presence of calcium chloride. 403.9 mg of calcium chloride dihydrate was dissolved in 99.4 ml of distilled water. 25.6 ml of thawed PLE and the calcium chloride solution were added to 180 ml of thawed prothrombin solution and the resulting mixture was stirred for 1 hour at 25°C to obtain conversion of the prothrombin to the active thrombin. pH was adjusted to 7.1-7.3 using dilute NaOH or dilute HCl. The resulting solution contained approximately 5.500 NIH units per ml. Prior to use this solution was diluted with 0.01M phosphate buffer to contain 1880 NIH units/ml. The final diluted activated thrombin solution contained 2.5 mM of CaCl_2 .

0.7 ml aliquots of the above thrombin solution were injected into Spongostan® haemostatic sponges followed by drying the injected sponges in an air drying oven for 4 hours at the following temperatures: 50, 65, 80, 91, 100, 110, 120, and 130°C. After drying the residual thrombin activity was measured in duplicate by the method described in Example 1. The results are summarized in Table 13 below:

Table 13

Residual thrombin activity in Spongostan® sponges injected with 1316 NIH units of thrombin activity and dried for 4 hours

5	<hr/>		
	Drying temperature, °C	Residual thrombin- activity, NIH units	% of initial activity
10	50	1223	93
	65	1208	92
	80	1208	92
	85	1223	93
	91	1198	91
	100	1093	83
	110	700	53
15	120	393	30
	130	243	18
<hr/>			

The thrombin activity remained essentially unaffected during drying at temperatures up till 91°C. From 100°C a decline of thrombin activity was observed. However, even at 130°C a proportion of 18% of the thrombin activity remained active.

CLAIMS

1. A haemostatic sponge comprising a porous structure of biologically absorbable, solid material, a haemostatically effective amount of
5 thrombin or a precursor therefor, and one or more thrombin-stabilizing agents, selected from naturally occurring amino acids, mono- or disaccharides, polyglycols, proteins, and mixtures thereof, said sponge having a total water content of below 50% by weight.
2. A haemostatic sponge according to claim 1 in which the biological-
10 ly absorbable, solid material is selected from gelatine, collagen, chitin, cellulose, polyglycolic acid, polylactic acid, and mixtures thereof.
3. A haemostatic sponge according to claims 2 in which the biologically absorbable solid material is gelatine.
- 15 4. A haemostatic sponge according to claims 3 in which the gelatine is denatured.
5. A haemostatic sponge according to claims 4 in which the gelatine is denatured by thermal treatment.
6. A haemostatic sponge according to claim 5 in which the gelatine is
20 thermally denatured by exposure to air at a temperature in the range of 100 - 160°C, preferably at a temperature of 150°C, the exposure time being 0.5 - 4 hours.
7. A haemostatic sponge according to claims 4 in which the gelatine is denatured by treatment with a protein denaturing compound, such as
25 an aqueous solution of formaldehyde, acids, bases, solvents, urea, and detergents.
8. A haemostatic sponge according to claim 1 in which the average content of thrombin is in the range of 0.1 - 300 NIH units per cm³, preferably 0.2 - 60 NIH units per cm³, in particular 1 - 40 NIH units
30 per cm³.

9. A haemostatic sponge according to claim 1 in which the naturally occurring amino acid is glycine, lysine, or arginine.
10. A haemostatic sponge according to claim 1 in which the mono- or disaccharide is selected from glucose, galactose, lactose, saccharose, maltose, and fructose.
11. A haemostatic sponge according to claim 1 in which the polyglycol is selected from polypropylene glycol and polyethylene glycol, preferably polyethylene glycol having a molecular weight in the range of 400 - 20,000, such as 6,000.
12. A haemostatic sponge according to claim 1 in which the protein is selected from serum albumin, egg albumin, gelatine, collagen, casein, keratin, and globulins.
13. A haemostatic sponge according to any of claims 1 - 12 in which the total content of thrombin-stabilizing agent or agents is in the range of 0.05 - 0.5 mg per NIH unit of thrombin, preferably 0.1 - 0.2 mg per NIH unit of thrombin.
14. A haemostatic sponge according to any of claims 1 - 13 which further contains a polyvalent alcohol, preferably selected from glycerol, sorbitol, mannitol, erythritol, ethylene glycol, and propylene glycol.
15. A haemostatic sponge according to any of claims 1 - 14 which further contains one or more buffering salts selected from alkaline metal acetates, alkaline metal citrates, alkaline metal phosphates or hydrogen phosphates, alkaline metal carbonates or hydrogen carbonates, alkaline metal succinates, imidazole, TRIS, and zwitteranionic buffering systems, and mixtures thereof.
16. A haemostatic sponge according to any of claims 1 - 15 which further contains sodium chloride in the range of 0.0006 - 2 mg, preferably 0.003 - 1.0 mg, in particular 0.005 - 0.2 mg per NIH unit of thrombin.

17. A haemostatic sponge according to any of claims 1 - 16 which further contains haemostatically effective amounts of one or more blood coagulation factors other than thrombin or a precursor therefor, preferably selected from Factor Va, Factor-VIIa, Factor VIIIa, Factor IXa, Factor Xa, Factor XIa, Factor XIIa, Factor XIIIa, and calcium ions.
18. A haemostatic sponge according to any of claims 1 - 17 which further contains an anti-fibrinolytic agent, preferably selected from aprotinin, pepstatin, leupeptin, antipain, chymostatin, ϵ -aminocaproic acid, tranexamic acid, and gabexate mesilate.
19. A haemostatic sponge according to any of claims 1 - 18 which further contains one or more antimicrobial agents selected from antibiotics, sulphonamides, antimycotic agents, antiviral compounds, and anti-infectives.
20. A haemostatic sponge according to any of claims 1 - 19 in which the total water content is below 50%, preferably below 30%, more preferably below 20%, in particular below 15%, more particularly 10%, and particularly preferred around 7% by weight.
21. A method for preparing a haemostatic sponge comprising a biologically absorbable, solid material, a haemostatically effective amount of thrombin, and one or more thrombin-stabilizing agents, said sponge having a total water content of below 50% by weight, comprising the following steps:
- 1) preparing a sponge comprising a porous structure of biologically absorbable, solid material;
 - 2) injecting into said sponge at a multiplicity of sites an aqueous solution of thrombin or a precursor therefor, one or more thrombin-stabilizing agents and optionally one or more buffering salts; and optionally sodium chloride; and optionally further blood coagulation factors; and optionally one or more anti-fibrinolytic agents; and optionally one or more antimicrobial agents; and
 - 3) air drying the sponge from step 2) at a temperature in the range of 30 - 100°C, preferably 40 - 55°C, such as 50°C, for

a time period sufficient to reduce the total water content to below 50%, preferably below 30%, more preferably below 20%, in particular below 15%, more particularly below 10%, and particularly preferred below around 7% by weight.

- 5 22. A method according to claim 21 in which the thrombin-stabilizing agent or agents are selected from naturally occurring amino acids, mono- or disaccharides, polyglycols, proteins, polyvalent alcohols, or mixtures thereof.
23. A method according to claim 21 in which the biologically absor-
10 bable, solid material is selected from gelatine, collagen, chitin, cellulose, polyglycolic acid, and polylactic acid.
24. A method according to claim 23 in which the biologically absor-
bable, solid material is gelatine.
25. A method according to claim 24 in which gelatine is denatured.
- 15 26. A method according to claim 25 in which the gelatine is denatured by thermal treatment.
27. A method according to claim 26 in which the thermal treatment comprises exposure of gelatine to air at a temperature in the range of 100 - 160°C, preferably at a temperature of 150°C, the exposure
20 time being 0.5 - 4 hours.
28. A method according to claim 25 in which the gelatine is denatured by treatment with a protein denaturing compound, such as an aqueous solution of formaldehyde, acids, bases, solvents, urea, and deter-
gents.
- 25 29. A method according to claim 21 in which the concentration of thrombin in the aqueous solution injected into the sponge is in the range of 500 - 2000 NIH units per ml.
30. A method according to claim 22 in which the concentration of amino acid in the thrombin solution is in the range of 0.01 - 0.1M.

31. A method according to claim 30 in which the concentration of amino acid is 0.05M.
32. A method according to claim 22 in which the concentration of mono- or disaccharides in the thrombin solution is in the range of
5 0.01 - 0.1M.
33. A method according to claim 32 in which the concentration of mono- or disaccharides is 0.05M.
34. A method according to claim 22 in which the concentration of polyglycol in the thrombin solution is in the range of 0 - 50% by
10 volume.
35. A method according to claim 34 in which the concentration of polyglycol is 20% by volume.
36. A method according to any of claims 34 and 35 in which the polyglycol is polyethylene glycol.
- 15 37. A method according to claim 22 in which the concentration of protein in the thrombin solution is in the range of 0 - 5% by weight.
38. A method according to claim 37 in which the concentration of protein is 3% by weight.
39. A method according to any of claims 22 - 38 in which the protein
20 is albumin.
40. A method according to any of claims 22 - 39 in which the total concentration of thrombin-stabilizing agents in the thrombin solution is in the range of 7 - 640 mg per ml, preferably in the range of
150 - 400 mg per ml.
- 25 41. A method according to claims 22 in which the polyvalent alcohol is present in a concentration in the range of 0.01 - 0.1M, preferably 0.05M.

42. A method according to any of claims 21 - 41 in which the thrombin solution contains sodium chloride within a concentration range of 0.5 - 1.5% by weight.
43. A method according to claim 42 in which the concentration of sodium chloride is 0.9%.
44. A method according to any of claims 21 - 43 in which the concentration of buffering salts in the thrombin solution is in the range of 0.001 - 0.1M.
45. A method according to claim 44 in which the concentration of buffering salts is 0.01M.
46. A method according to any of claims 21 - 45 wherein the stabilized thrombin solution being injected has a viscosity sufficiently high to ensure that the porous structure of the sponge is substantially preserved during the injection.
47. A method according to any of claims 21 - 45 in which part of the aqueous solvent is replaced by a monovalent alcohol, such as methanol, ethanol, propanols, or butanols.
48. A method according to claim 47 in which the monovalent alcohol is ethanol, the ethanol concentration being in the range of 0.1 - 40% by volume.
49. A method according to claim 21 in which the injection into the sponge material of an aqueous solution of thrombin or a precursor therefor containing one or more thrombin-stabilizing agents, and optionally one or more buffering salts; and optionally sodium chloride; and optionally further blood coagulation factors; and optionally one or more anti-fibrinolytic agents; and optionally one or more antimicrobial agents is carried out by means of a single or preferably multiple injection device delivering preset quantities of said stabilized thrombin solution into said sponge material.

50. A method according to claim 49 in which the quantity of thrombin solution delivered at each injection site is in the range of 0.005 - 0.2 ml, preferably in the range of 0.01 - 0.5 ml, in particular 0.03 ml.
- 5 51. A method according to any of claims 49 - 50 in which the injection needle points are placed at intervals of 5 - 20 mm, preferably 10 - 15 mm, on the same or on staggered planes.
52. A method according to claim 21 in which the drying process is carried out at atmospheric pressure.
- 10 53. A method according to claim 21 in which the drying process is carried out at air pressures below atmospheric pressure.
54. A method according to claim 21 in which the original shape of the sponge material subsequent to the injection of the stabilized thrombin solution is modified by compression or rolling into sheets or
15 by extrusion under reduced pressure, the extrusion resulting in a three-dimensional expansion of the sponge.
55. A method according to any of claims 21 - 54 in which a plate of haemostatic sponge according to any of claims 1 - 19 subsequent to injection of the stabilized thrombin solution and drying is cut into
20 surgically suitably sized haemostatic sponge units.
56. A method according to claim 55 in which surgically suitably sized sponge units are packaged in a hermetically sealed package.
57. A method according to any of claims 55 - 56 in which hermetically sealed packages of haemostatic sponges are sterilized by β - or γ -
25 irradiation at a dosage of 10 - 50 kGy, preferably in the range of 20 - 40 kGy.
58. Use of a haemostatic sponge according to any of claims 1 - 20 as a haemostatic adjunct in medical, veterinary, or dental surgery.

59. Use of a haemostatic sponge according to any of claims 1 - 20 for the preparation of a haemostatic adjunct to be used in medical, veterinary, or dental surgery.

60. A method for arresting bleeding comprising the application to the
5 site of bleeding of a haemostatic sponge according to any of claims
1 - 20.

INTERNATIONAL SEARCH REPORT

International Application No **PCT/DK 90/00114**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC⁵: A 61 L 15/44, A 61 L 15/64, A 61 L 15/32		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
IPC⁵	A 61 L	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT *		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	EP, A, 0277096 (WARNER LAMBERT) 3 August 1988 see example 4; page 3, lines 8-9, 55-59; claims 1,5	1-3,8,13-16, 19,58-60
Y	--	7,9,17,18
A	EP, A, 0221700 (WARNER LAMBERT) 13 May 1987 see page 4, lines 1-8	1-3,8,11,13- 16,19
Y	CH, A, 267523 (FERROSAN) 16 June 1950 see page 2, lines 40-51	7
Y	GB, A, 2081090 (APPLIED MEDICAL DEVICES) 17 February 1982 see claim 3	9
	--	
	./.	
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
21st August 1990	21 SEP 1990	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	Mme N. KUIPER	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y FR, A, 2422407 (UNITIKA)
9 November 1979
see claims 7-12,22,24
cited in the application

17,18

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 60... because they relate to subject matter not required to be searched by this Authority, namely:
PCT Rule 39.1(iv): methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods
2. ☐ Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this International application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

DK 9000114
SA 36903

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 14/09/90
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0277096	03-08-88	AU-A- 1039488	04-08-88
		JP-A- 63192723	10-08-88
		ZA-A- 8800227	30-06-88
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		AU-B- 586897	27-07-89
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		US-A- 4265233	05-05-81

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